Applied Environmental Microbiology Core: Rapid Deduction of Stress Response Pathways in Metal/Radionuclide Reducing Bacteria

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Virtual Institute of Microbial Stress and Survival

http://vimss.lbl.gov

Abstract

Field Studies

Sulfate-reducing bacteria in NABIR FRC: Sediment samples from different depths at the NABIR Field Research Center in the background, Areas 1, 2, and 3 sites have been used for the enrichment of sulfate-reducing microorganisms. Sulfate-reducing enrichments have been positive for sediments in Areas 1 and 2 when lactate or acetate were used as electron donors, and some of the enrichments differ in the capacity to reduce cobalt, chromium, and uranium. Groundwater enrichments from Areas 1, 2, and 3 all displayed sulfate reduction with different electron donors (lactate, butyrate, acetate, pyruvate) and these enrichments could also reduce iron, cobalt, and chromium. Subsurface sediments from the wells FWB-107 (13.2 m) and FWB-109 (15.4 m) in Area 3 were serially diluted in a basal salts medium that contained lactate and ethanol with different electron acceptors. The results suggested that in the sampled sediments (13 to 15 m) nitrate-reducers were approximately 3500 to 5400 cells/g, iron-reducers 50 to 1700 cells/g, and sulfate-reducers 240 to 1100 cells/g. 16S rRNA sequence of the predominant population (25%) of the 10-2 sulfate-reducing dilution had 88% sequence identity with *Desulfosporosinus* Blif. Subpopulations that had 95% to 97% sequence identityof 16S rRNA with *Desulfosporosinus orientis* constituted an additional 37% of the library. Other clones had 98% sequence identity with *Clostridium chromoreductan*s.

Clone libraries of DNA isolated from NABIR FRC. Since genes for stress response pathways are clustered on chromosomal DNA fragments and generally vary in length from 20-40 kb, it is essential to clone large DNA to capture entire pathways. We have developed new effective DNA extraction methods and vector/host systems that allow stable propagation of large DNA fragments in *E. coli*. Processed environmental samples are embedded in agarose noodles for protein digestion and release of high-molecular-weight DNA. In stressed environments, organism concentrations are often very low, so we have developed a method for increasing the concentration of large DNA by amplification with a phage polymerase. After amplification, the DNA is partially digested with restriction enzymes, and size-selected by agarose gel electrophoresis. It is then ligated to fosmid arms and packaged into phage lambda particles that are used to infect *E. coli*. The microbial diversity of the libraries is determined with Terminal Restriction Fragment Polymorphism (T-RFLP). Large fragment DNA has been extracted and amplified from 15 NABIR FRC samples (comprising 3 areas at various depths). Small insert DNA libraries have been constructed from most of these samples, and large-insert DNA libraries are in various stages of construction. T-RFLP and DNA sequencing are being used to quality control the resulting libraries.

Enrichments. Ten *Desulfovibrio* strains were isolated from lactate-sulfate enrichment of sediment taken from the most Zn contaminated region of Lake DePue, IL. Their 16S rRNA and dsrAB genes were amplified and sequenced. They all were identical to each other and virtually identical to the corresponding genes from *D. vulgaris* Hildenborough. One mismatch was observed in the16S rRNA gene and one in dsrAB. Different fragment patterns confirmed that the DePue isolates were similar but not identical to *D. vulgaris* Hildenborough. Pulse field electrophoretic analysis of I-CeuI digests revealed that both isolates had five rRNA clusters, the same as *D. vulgaris* Hildenborough. However, the length of one chromosomal segment in the DP isolates was considerably shorter than the corresponding fragment from *D. vulgaris* Hildenborough, suggesting the presence of a large deletion in the genomes of the isolates (or insertion in *D. vulgaris* Hildenborough).

Culture and Biomass Production

Defined Media – Growth. A defined medium for optimal growth and maximum reproducibility of *Desulfovibrio vulgaris* was developed for biomass production for stress response studies. The medium was optimized by evaluating a variety of chemical components, including the removal of yeast extract, excess sulfate, and Fe, and redox conditions to optimize cell density and generation times, and to reduce lag times. Growth was monitored using direct cell counts, optical density, and protein concentration. The generation time for *D. vulgaris* in the original Baar's medium was 3 h, reaching a maximum density of 10^8 cells/ml and 0.4 $OD_{600\ nm}$. The generation time for *D. vulgaris* on LS4D was 5 h, with a maximum cell density of 10^9 cells/ml and a 0.9-1.0 $OD_{600\ nm}$. LS4D is well suited for the monitoring protocols, as well as the equipment and large-scale processing needed for biomass production.

Dual culture systems. Co-cultures of two different *Desulfovibrio* species (*Desulfovibrio vulgaris* Hildenborough and *Desulfovibrio* sp.PT2) syntrophically coupled to a hydrogenotrophic methanogen (*Methanococcus maripaludis*) on a lactate medium without sulfate has been established and characterized. No appreciable growth was observed in 50 mM lactate for single-organism cultures. Following optimization of the ionic composition (MgCl2 and NaCl) of the medium, stable co-cultures were established having generation times of 25h-1 and 35 h-1 for *D. vulgaris* and *Desulfovibrio* sp. PT2 co-cultures respectively. Both co-cultures degraded lactate to acetate, methane, and carbon dioxide. No other organic acids were detected during the course of experiments. Approximately 1mol of acetate and 1mol of methane was produced from two mole of lactate by both co-cultures during most active period of growth. The stability of established methanogen-SRBs co-cultures (*Desulfovibrio vulgaris* or *Desulfovibrio* sp. PT2 with *M. maripulidis*) was confirmed by serial transfer (six times).

Biofilm reactors. Initial characterization of *Desulfovibrio vulgaris* growth as a biofilm was evaluated using a 600ml biofilm reactor containing 3mm glass beads as growth substratum and the B3 culture medium (16mM lactate and 28 mM sulfate). The ratio of flow rates through an internal recirculation loop to influent was maintained at 100:1, evaluating two different influent flow rates (0.5ml/min or 30ml/hr). Formation of a loose biofilm was associated with significant gas accumulation within the reactor. The system in now being modified to incorporate a gas trap in the re-circulation loop.

FairMenTec (FMT) Chemostat. A pilot run with *Desulfovibrio vulgaris* Hildenborough in the FMT bioreactor in chemostat mode was completed. The bioreactor was operated using the LS4D medium with 45mM lactate, 50 mM sulfate, and Ti-citrate at 1/3 standard formulation (subsequent batch cultures have shown improved growth with further reduction of the Ti-citrate to 1/6 standard formulation). Varying flow rates and medium compositions were evaluated.

Oxygen Stress Experiments

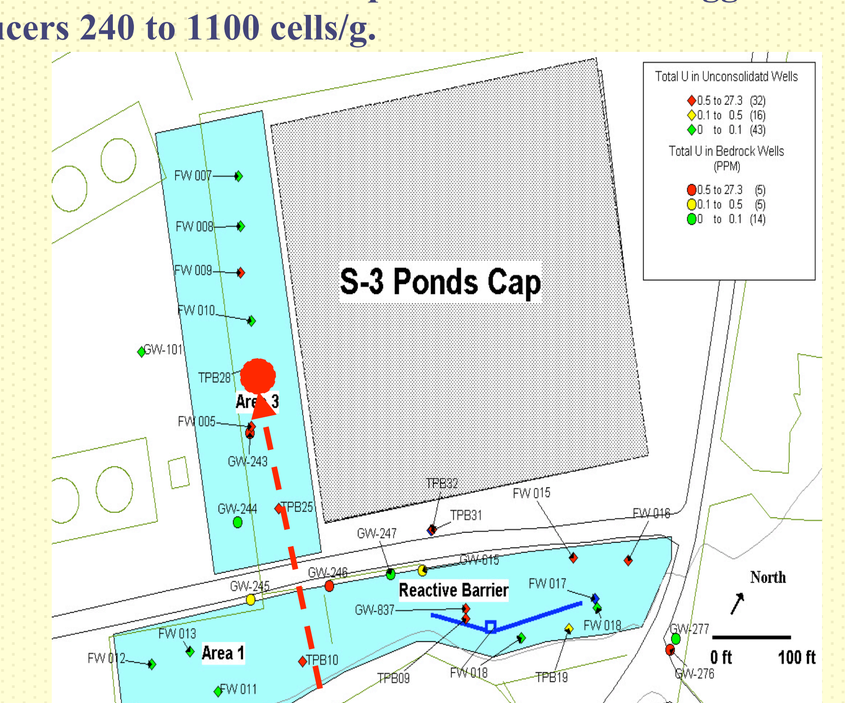
Protocols. Since episodic exposure to air or oxygenated ground water is common at contaminated sites, we decided to focus on oxygen stress of *D. vulgaris* for our initial studies. To accommodate all the investigations that would require simultaneous harvesting of biomass for studies on proteomics, transcriptomics, metabolomics and phenotypic studies a batch culture system was developed for 2000 ml cultures that could be sparged with nitrogen or air to control stress in water baths using rigorous quality control on culture age, sampling, defined media, chain of custody, and harvesting times and techniques.

Phenotypic Responses. Desulfovibrio vulgaris enters a new phenotypic state when confronted with a sudden influx of oxygen. Using SEM and TEM microscopy we observed that during the first 24-72 h of exposure to air *D. vulgaris* cells are negatively aerotactic, gradually they loose their flagella, and begin to elongate. By 20 days exposure they are 3-4 times larger and have a well developed exopolysaccharide sheath. At all times the cells were viable and recovered when put back under anaerobic conditions. Real-time analysis using Synchrotron Fourier Transform Infrared Spectromicroscopy enabled us to determine quantitative changes in peptides and saccharides in the living cells during exposure to air, thus providing the exact timing of cell changes in the stress response. During the early phase of the exposure, we observed decreases in total cellular proteins as well as changes in the secondary structures of proteins that are indicative of the changing of the local hydrogen-bonding environments and the presence of granular protein. During the late phase of the exposure, we observed the production of polysaccharides, concomitant with the production of the external sheath. The S-FTIR also demonstrated that the cells were viable within the sheath at 20 days exposure. Phospholipid fatty acid (PLFA) analysis confirmed that no biomass was loss during air sparging of stationary phase cells. In addition, no change in the PLFA patterns were observed during air sparge, indicating neither cell growth nor death occurred. The PLFA extraction is being developed as a method for routine monitoring of cultures during biomass production and stress studies. Databases of lipid signatures of *D. vulgaris* during various growth conditions are being developed to augment the information produced from other VIMSS collaborators on proteomics and functional genomics.

Field Studies

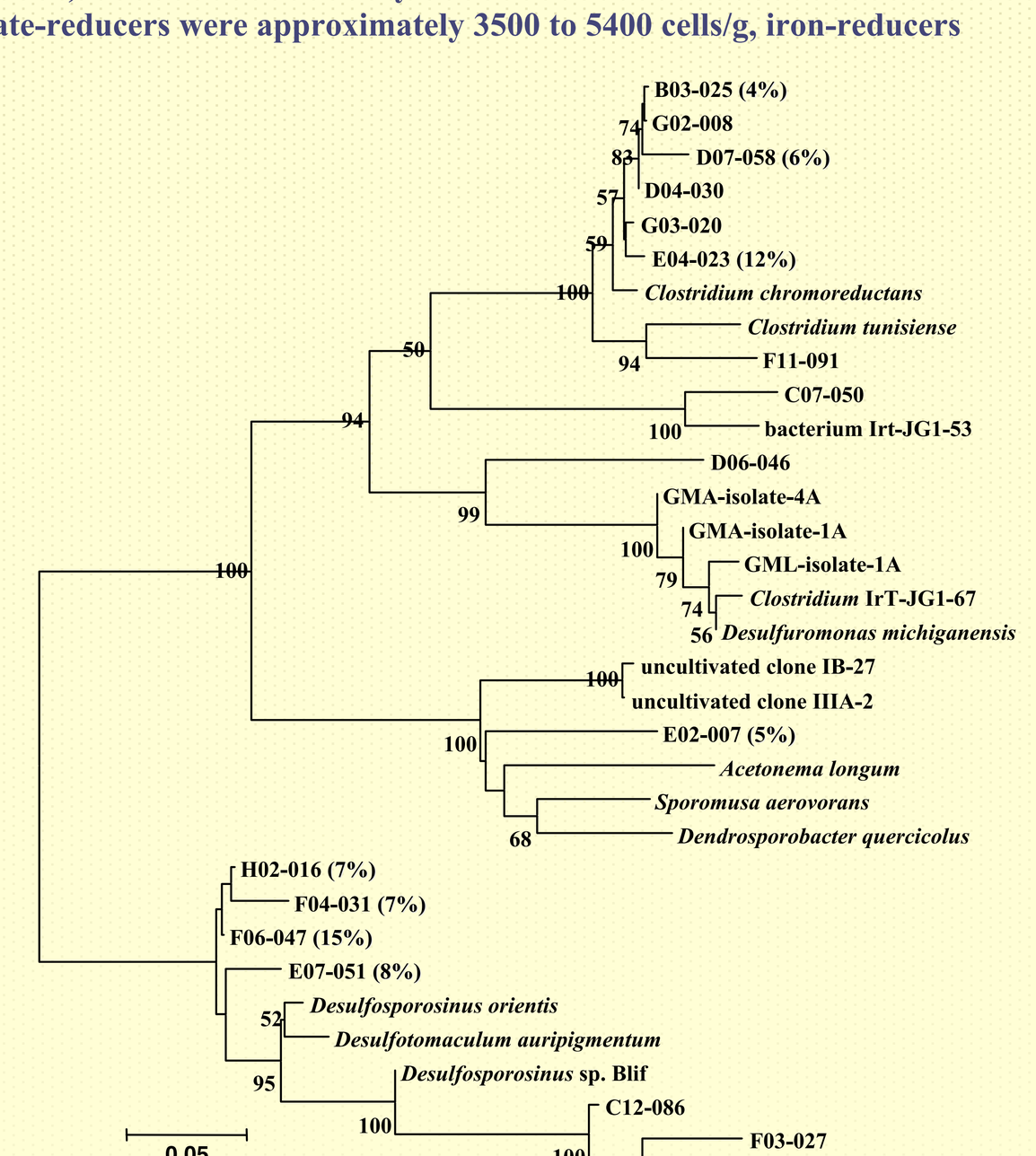

Sulfate-reducing bacteria: Sediment samples from different depths at the NABIR Field Research Center in the background, Areas 1, 2, and 3 sites have been used for the enrichment of sulfate-reducing microorganisms. Sulfate-reducing enrichments have been positive for sediments in Areas 1 and 2 when lactate or acetate were used as electron donors, and some of the enrichments differ in the capacity to reduce cobalt, chromium, and uranium. Groundwater enrichments from Areas 1, 2, and 3 all displayed sulfate reduction with different electron donors (lactate, butyrate, acetate, pyruvate) and these enrichments could also reduce iron, cobalt, and chromium. Subsurface sediments from the wells FWB-107 (13.2 m) and FWB-109 (15.4 m) in Area 3 were serially diluted in a basal salts medium that contained lactate and ethanol with different electron acceptors. The results suggested that in the sampled sediments (13 to 15 m) nitrate-reducers were approximately 3500 to 5400 cells/g, iron-reducers 50 to 1700 cells/g, and sulfate-reducers 240 to 1100 cells/g.

Subsurface sediments from the wells FWB-107 (13.2 m) and FWB-109 (15.4 m) in Area 3 were serially diluted in a basal salts medium (NaCl, $NaHCO_3$, NH_4Cl, minerals, 99%:1% N_2/CO_2) with 5 mM lactate and 5 mM ethanol. The dilutions were provided with nitrate, Fe(III)-citrate, or sulfate and incubated anaerobically at approximately 18 to 20°C. The results are summarized in the table below:



	NO_3	$Fe^{(III)}$	SO_4
FWB-107 (13.2 m)	3500 cells/g	46 cells/g	240 cells/g
FWB-109 (15.4 m)	5400 cells/g	1700 cells/g	1100 cells/g

Community analysis of sulfate-reducing enrichment from FW-109. The predominant population comprised approximately 25% of the sampled diversity and had 88% sequence identity with *Desulfosporosinus* Blif. Sub-populations that had 95% to 97% sequence identity with *Desulfosporosinus orientis* constituted for an additional 37% of the library. The clone E04-023 constituted just over 10% of the library, and had 98% sequence identity with *Clostridium chromoreductans*. A small fraction of the enrichment community (5%) appeared to have only 87% sequence identity with previously uncultivated clones that originated from a chlorobenzene-degrading community.



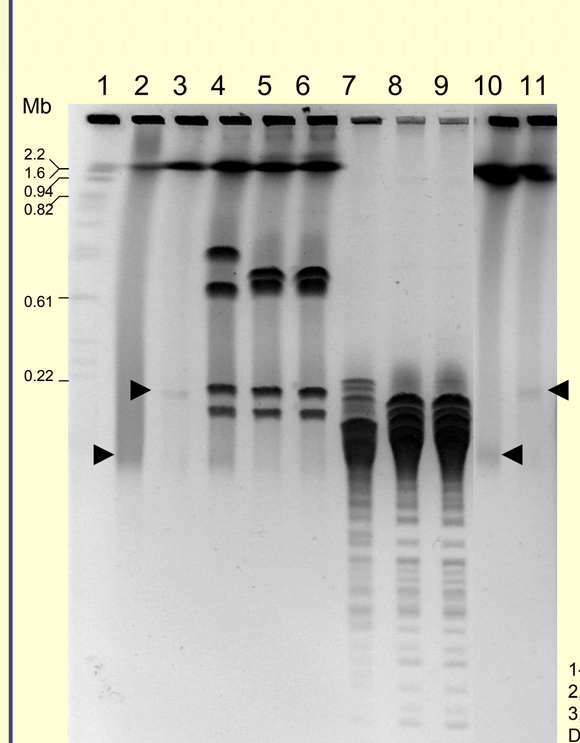
Clone libraries. Since stress response pathways are clustered on chromosomal DNA fragments and generally vary in length from 20-40 kb, it is essential to clone large DNA fragments to capture entire pathways. Large fragment DNA has been extracted and amplified from 15 NABIR FRC samples (comprising 3 areas at various depths). Small insert DNA libraries have been constructed from most of these samples, and large insert DNA libraries are in various stages of construction. T-RFLP and DNA sequencing are being used to quality control the resulting libraries.

Sample ID	Sample Name	Core Segment	Core Depth	Comments	Small Insert DNA Conc. Pre-Ampli	Small Insert DNA Conc. Post-Ampli	Small Insert Library	Large Insert Library
Background								
FB609-01-00	B-Top	24-43" long	180-234"	Background,	5ng/µl	278ng/µl	Yes	Yes
	B-Bottom			Anaerobic	4ng/µl	209ng/µl	Yes	Yes
Area 1								
FB060-01-27	1-Top	27-46" long	180-228 "	<25ppm U238	5ng/µl	140ng/µl	Yes	Yes
	1-Bottom			Anaerobic	5 ng/µl	206ng/µl	Yes	
FB060-01-00	1aL	0-25" long	180-228"		~	~	Yes	QC
Area 2								
FB052-01-12	2-Top	12-36" long	245-300"	<200ppm U238	5ng/µl	246ng/µl	Yes	
	2-Bottom			Anaerobic	5 ng/µl	212ng/µl	Yes	
FB052-01-00	2aL	12-36" long	245-300"		~	~	Yes	QC
	2bL				~	~	Yes	QC
Area 3								
FB056-01-34	3-Top	34-58" long	144-198"	<25ppm U238	2ng/µl	179ng/µl	Yes	Yes
	3-Bottom			Anaerobic	2ng/µl	263ng/µl	Yes	Yes
	3aL				~	~	Yes	QC
	3bL				~	~	Yes	QC

Notes:
Pre-Ampli = DNA conc. pre-amplification with DNA polymerase
Post-Ampli = DNA conc. post-amplification with DNA polymerase
QC = Library is constructed and is in QC for sequence analysis of inserts.

OTU number	OTU designation	Representative organism	Control	Lactate Stimulated*
02280401010000.4114	Dhb.retbaense_subgroup soil PBS-21	*Desulfohalobium retbaense*	nd	3234
02280401040100.683	Dsv.halophilus_subgroup Desulfovibrio sp. Ac5.2	*Desulfovibrio halophilus*	nd	2560
02280401040500.4661	Dsv.aminophilus_subgroup clone R2b32	*Desulfovibrio aminophilus*	nd	2772
02280401040600.2471	Dsv.africanus_subgroup delta clone:KB47	*Desulfovibrio africanus*	nd	2640
02280401041100.2700	Dsv.desulfuricans_subgroup delta clone:Rs-N31	*Desulfovibrio desulfuricans*	nd	2921

* Average difference, Sulfate reducing bacteria detected using the Affymetrix GeneChip high-density oligonucleotide array. NABIR FRC Area 2 Sediments



1- Size markers (*Saccharomyces cerevisiae* chromosomes); 2, 10 – *D. vulgaris Hildenborough* (DvH) undigested genomic DNA; 3,11- L. DePue isolates DP4 and DP8 undigested genomic DNA; 4- DvH DNA digested with I-CeuI; 5,6- DP4 and DP8 DNA digested with I-CeuI; 7- DvH DNA digested with NotI; 8,9- DP4 and DP8 DNA digested with NotI; Arrows indicate extrachromosal DNA

Enrichments. Seven *Desulfovibrio* strains were isolated from lactate-sulfate enrichment of sediment taken from the most contaminated region of Lake DePue, IL. Their 16S rRNA and dsrAB genes were amplified and sequenced. They all were identical to each other and virtually identical to the corresponding genes from *D. vulgaris* Hildenborough. One mismatch was observed in the16S rRNA gene and one in dsrAB. Different fragment patterns confirmed that the DePue isolates were similar but not identical to *D. vulgaris* Hildenborough. Pulse field electrophoretic analysis of I-CeuI digests revealed that both isolates had five rRNA clusters, the same as *D. vulgaris* Hildenborough. However, the length of one chromosomal segment in the DP isolates was considerably shorter than the corresponding fragment from *D. vulgaris* Hildenborough, suggesting the presence of a large deletion in the genomes of the isolates (or insertion in *D. vulgaris* Hildenborough).

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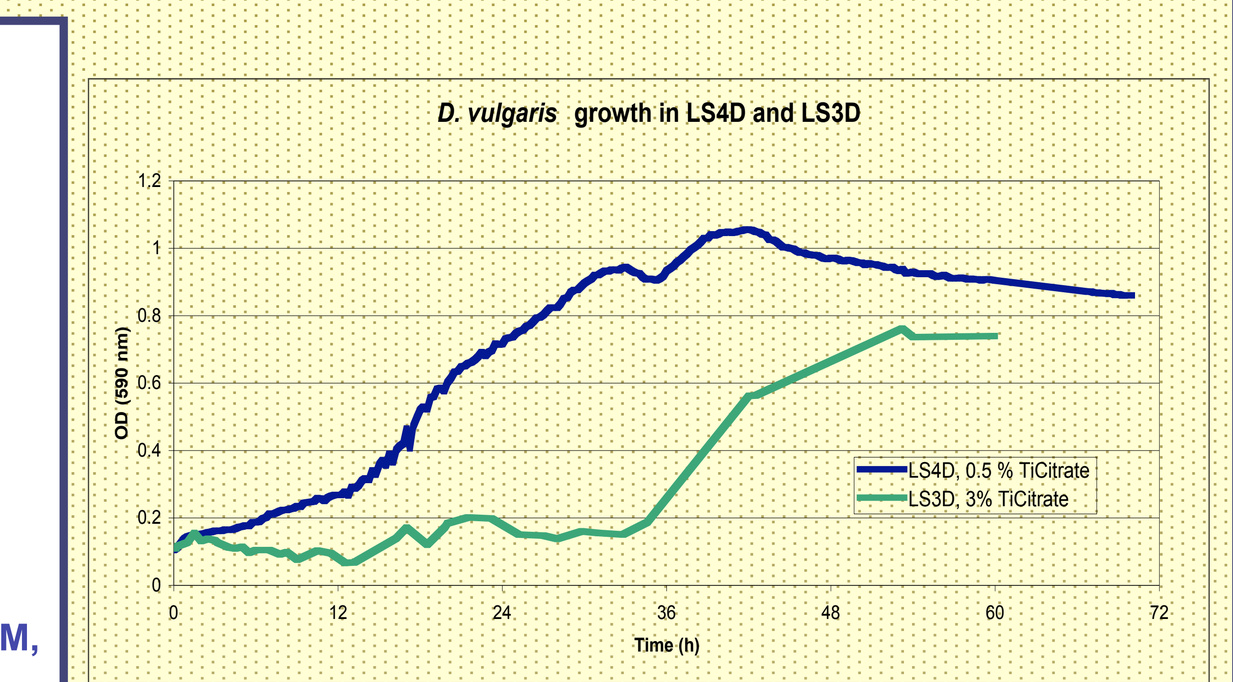
Compound	Molarity	ml/L stock	Supplier	Catalog #	Molarity in Medium	Storage
Degassed ddwater[1]		810 ml				Anaerobic
Sodium sulfate	0.5M	100 ml	Fisher	S421-500	50 mM	Anaerobic
Sodium lactate[2]	2M	30 ml	Fisher	S326-500	60 mM	4°C
Magnesium chloride	1M	8 ml	Fisher	M33-500	8.0 mM	Anaerobic
Ammonium chloride	4M	5 ml	Fisher	A687-500	20 mM	Anaerobic
Potassium phosphate	1M	2.2 ml	Fisher	P290-500	2.2 mM	Anaerobic
Calcium chloride	1M	0.6 ml	Fisher	C79-500	0.6 mM	Anaerobic
Thauers vitamins	10X	1 ml	Stock below			-20°C
Trace minerals		12.5 ml	Stock below			4°C
PIPES	1M	30 ml	Fisher	BP304-500	30 mM	Anaerobic
Resazurin (0.1%)[3]	0.1%	0.64 ml	Sigma	R7017	0.064 µM	Anaerobic
Sodium Hydroxide (Used to adjust pH of medium to 7.20)	5N	~ 1.8-2.2 ml	Sigma	S8045	10 mN	
Titanium Citrate (Added to medium before inoculation)		5 ml	Stock below			

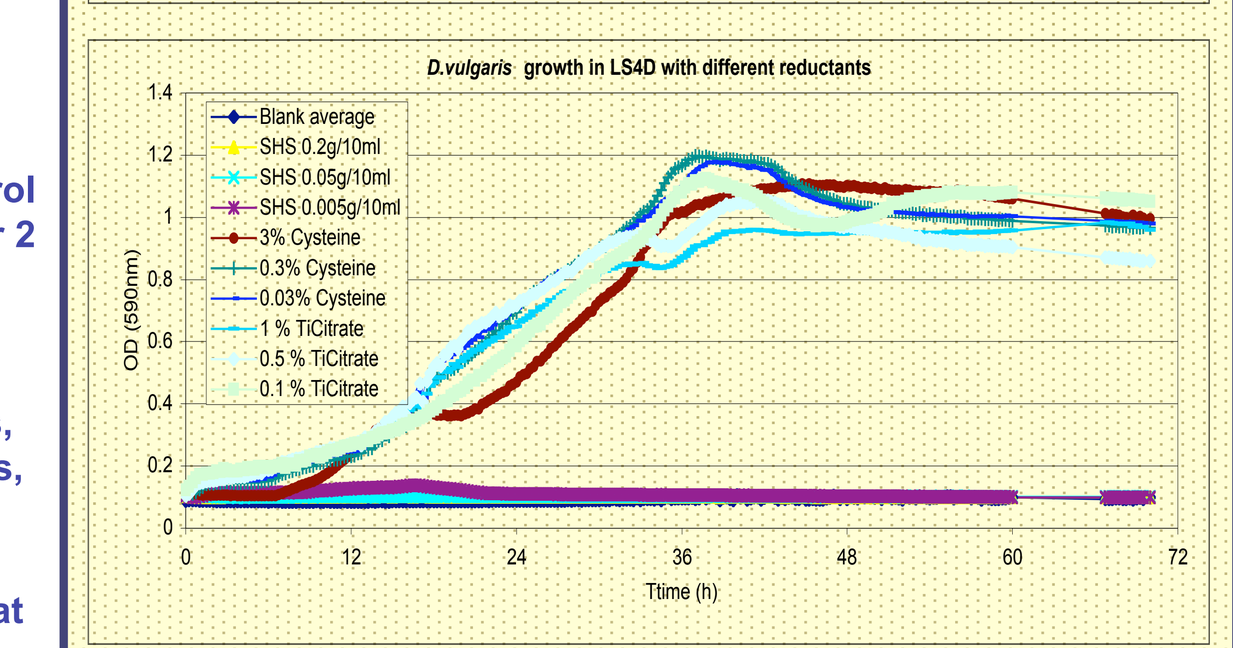
Phenotype parameters

- Metabolic – 750+ different tests
  - Carbon Pathways
  - P metabolism
  - N metabolism
  - S metabolism
  - Biosynthetic Pathways
  - Osmotic and Ion effects
  - pH effects
- Inhibitory – 239 different agents – to test sensitivity to chemicals
  - Antibiotics
    - Penicillin, amoxicillin, etc.
  - Antibacterial
    - Sodium azide
  - Toxic ions
    - Bromate, cyanide
  - Toxins
    - Atropine, cresol

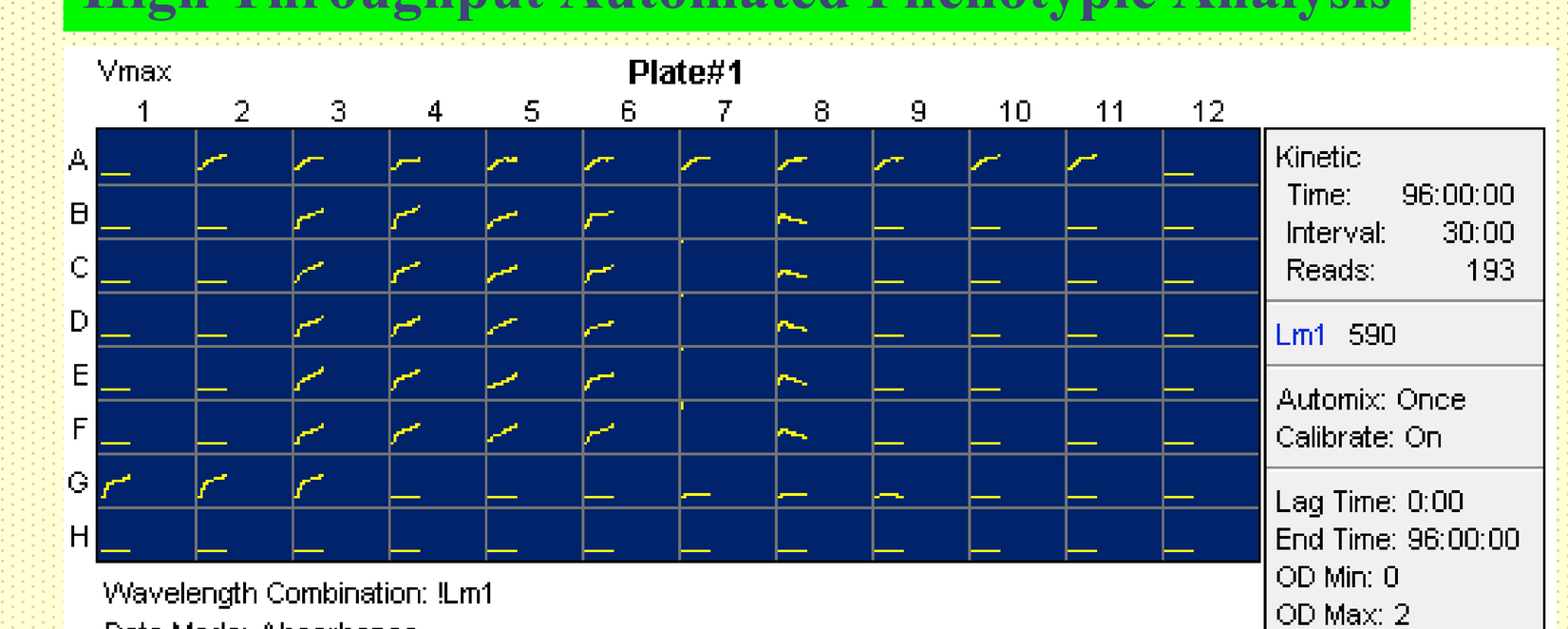
Biomass Production Core Activities

- Maintenance and archiving of *D. vulgaris* experimental strain (ATCC 29579) to minimize 'culture' drift from multiple transfers
- Develop and define growth conditions for stress studies using defined media (1000's of growth curves using automated systems)
- QA/QC on all media components and culture conditions, e.g. pH, DO, temperature, containers, anaerobic chamber, etc.
- Physiological and Morphological typing: PLFA, sFTIR, AODC, Fluorescent Antibody (O&H),Omnilog (Phenotype Microarray), SEM, TEM, x-ray, chemotaxis, protein, optical density, lactate/acetate, sulfate, etc.
- Develop and validate large scale production of biomass under sterile, anaerobic, reproducible conditions for simultaneous comparison of control and stress. Currently up to 2 L in triplicate under 2 conditions. Expanding to multiple 4 L no-metal, anaerobic chemostats.
- Develop techniques for stressing cells that minimize other stress responses: log phase cells, sparge with N and air for comparison of air stress, special porous tubing to maximize DO saturation from air.
- Develop sampling and processing techniques that minimize contamination, processing, shipping, and maximize sample quality and quantity for simultaneous sampling and processing for 5 different labs.





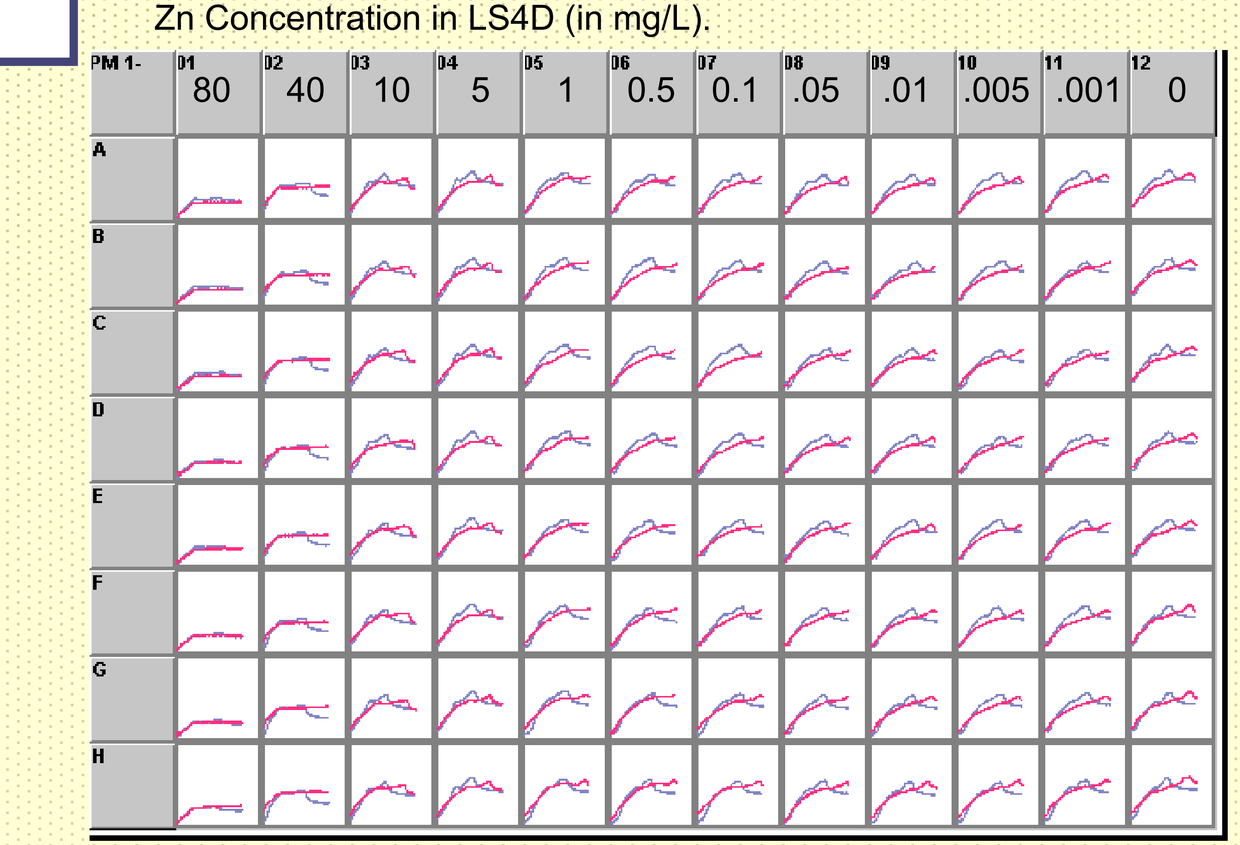
High Throughput Automated Phenotypic Analysis



Example Multiple Media Growth Curve

Phenotype Microarray Omnilog System

Zn Concentration in LS4D (in mg/L).

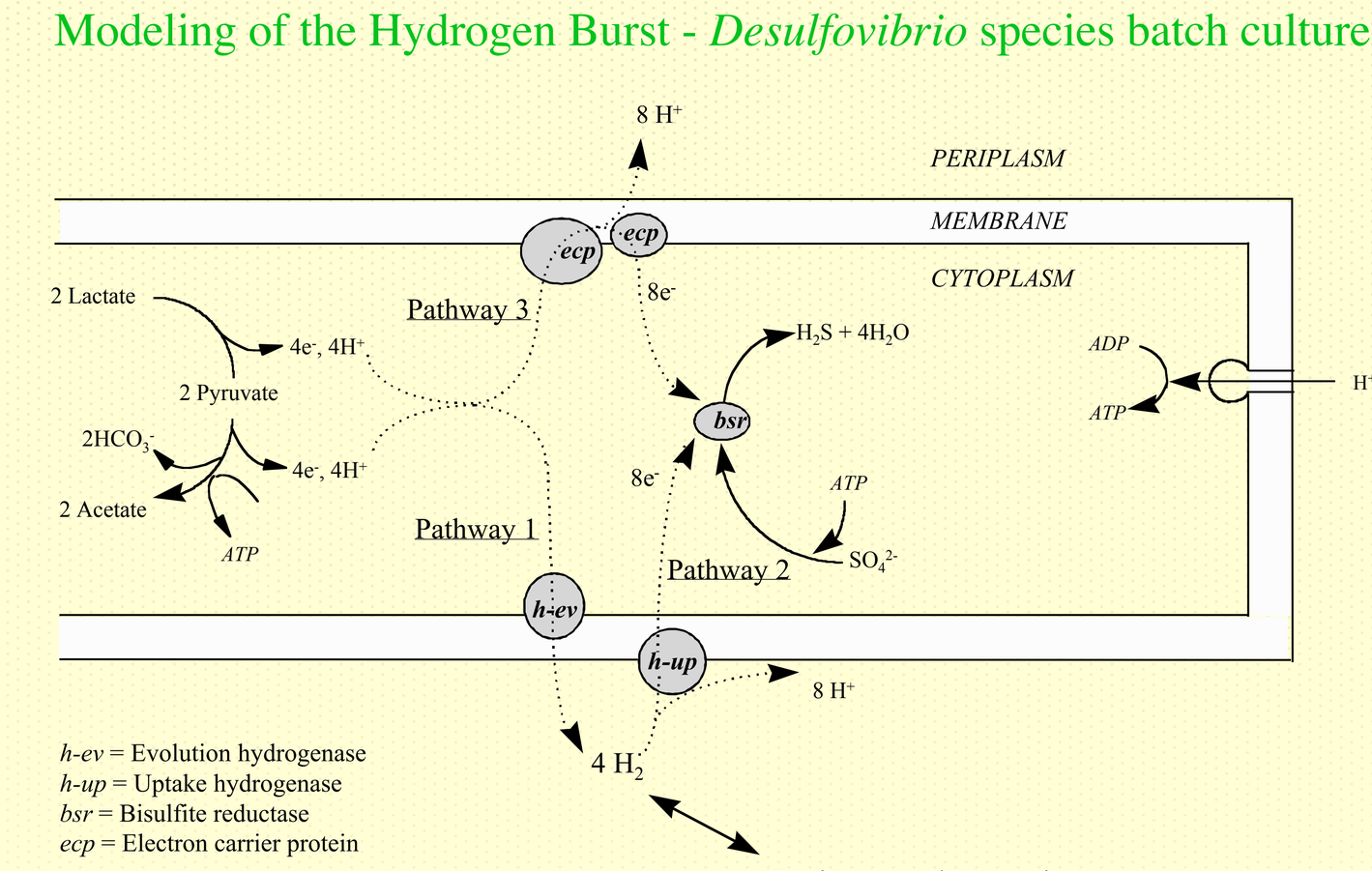


Desulfovibrio vulgaris, Hildenborough (blue trace)
DP9 strain from Lake DePue sediments (pink trace)

Bioreactors and Dual Culture

Dual culture systems. Co-cultures of two different *Desulfovibrio* species (*Desulfovibrio vulgaris* Hildenborough and *Desulfovibrio* sp.PT2) syntrophically coupled to a hydrogenotrophic methanogen (*Methanococcus maripaludis*) on a lactate medium without sulfate has been established and characterized. No appreciable growth was observed in 50 mM lactate for single-organism cultures. Following optimization of the ionic composition (MgCl2 and NaCl) of the medium, stable co-cultures were established having generation times of 25h-1 and 35 h-1 for *D. vulgaris* and *Desulfovibrio* sp. PT2 co-cultures respectively. Both co-cultures degraded lactate to acetate, methane, and carbon dioxide. No other organic acids were detected during the course of experiments. Approximately 1mol of acetate and 1mol of methane was produced from two mole of lactate by both co-cultures during most active period of growth. The stability of established methanogen-SRBs co-cultures (*Desulfovibrio vulgaris* or *Desulfovibrio* sp. PT2 with *M. maripulidis*) was confirmed by serial transfer (six times).

Modeling of the Hydrogen Burst - *Desulfovibrio* species batch culture



Lactate as electron donor; no electron acceptor

$$2CH_3CHOHCOO^- + 4H_2O \rightarrow 2CH_3COO^- + 2H^+ + 2HCO_3^- + 4H_2$$
$$\Delta G^{0\prime} = -8.4\ kJ\ (SRB\ alone)$$

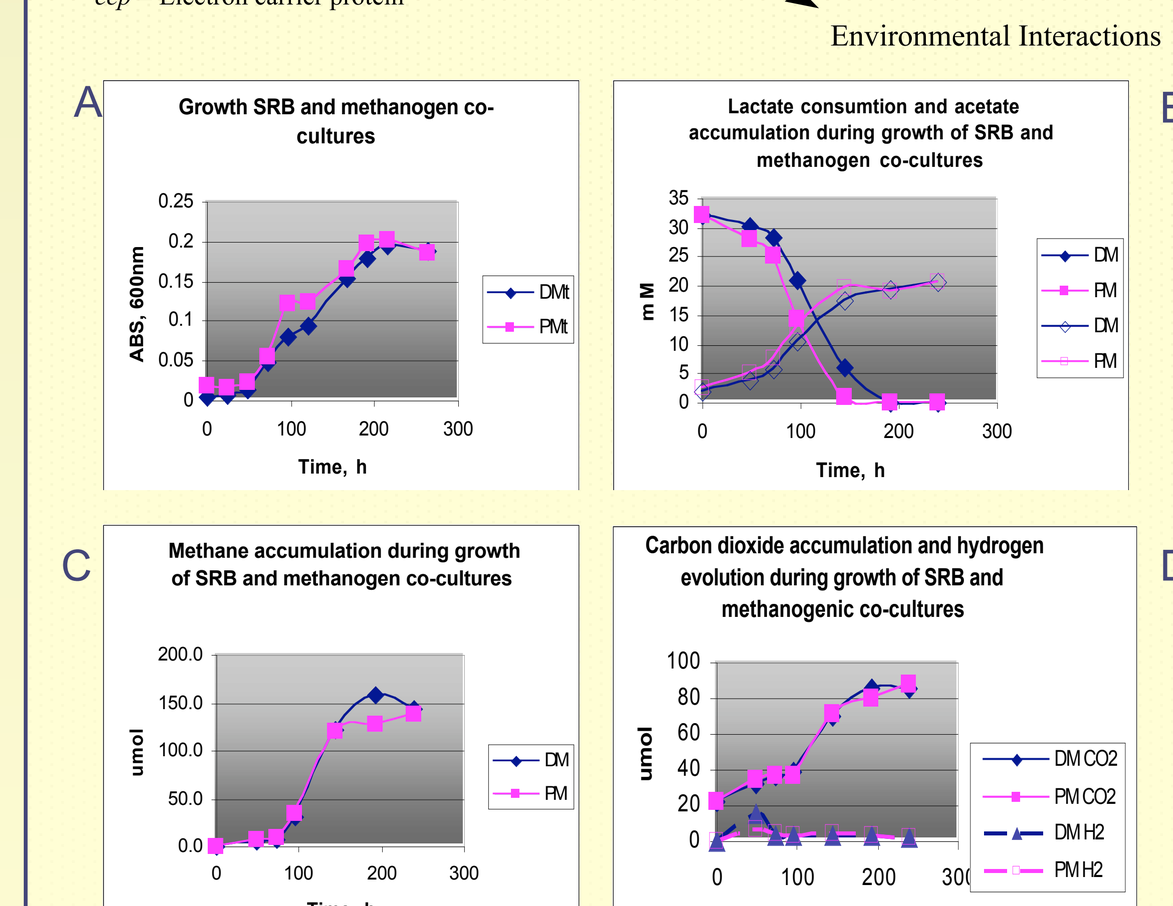
Hydrogen as electron donor; CO_2 as electron acceptor

$$4H_2 + H^+ + HCO_3^- \rightarrow CH_4 + 3H_2O$$
$$\Delta G^{0\prime} = -135.6\ kJ\ (Methanogen\ alone)$$

Combined equations (syntrophic growth)

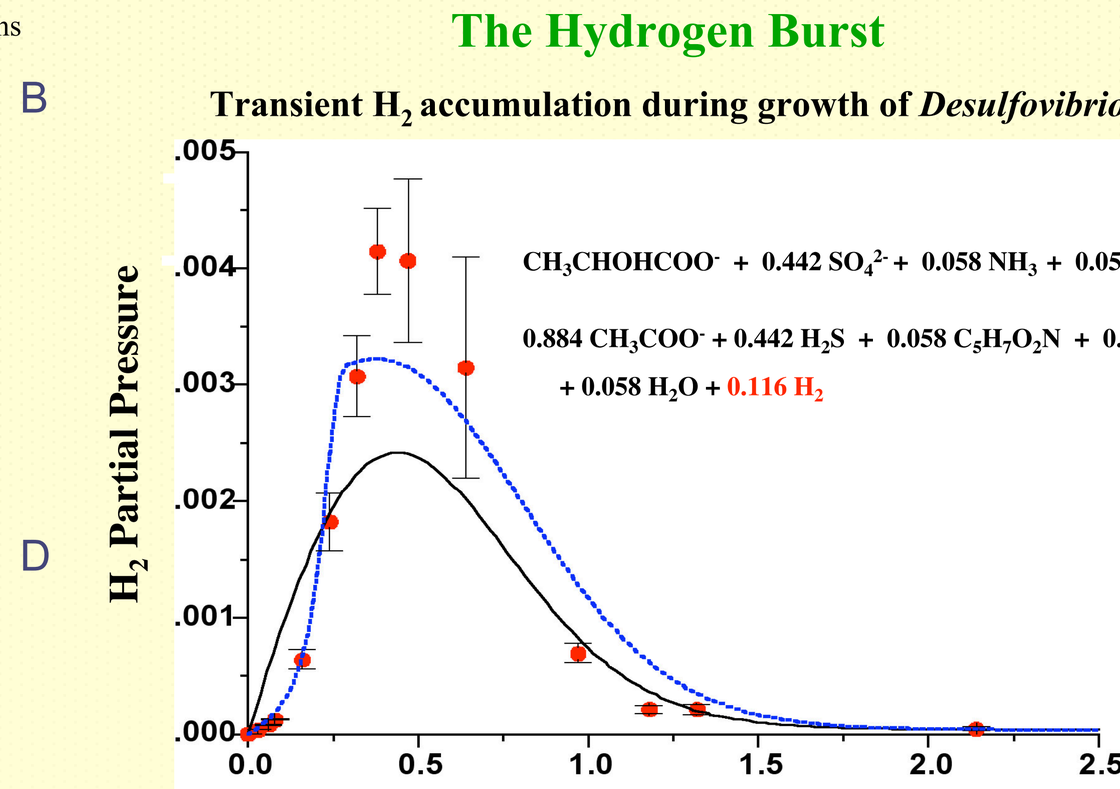
$$2CH_3CHOHCOO^- + H_2O \rightarrow 2CH_3COO^- + CH_4 + H^+ + HCO_3^-$$

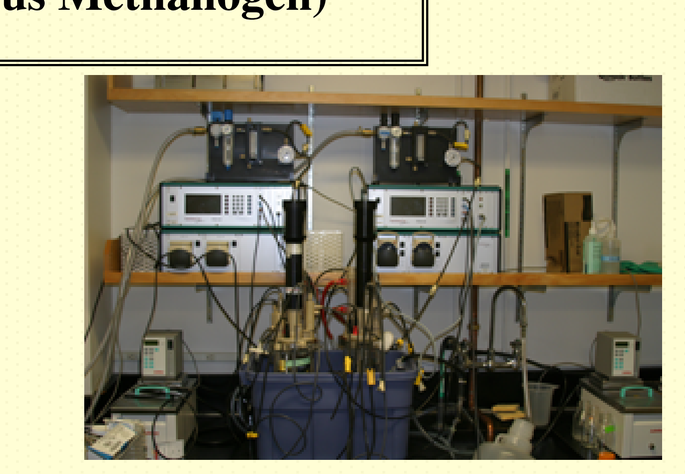
$$\Delta G^{0\prime} = -144.0\ kJ\ (SRB\ plus\ Methanogen)$$

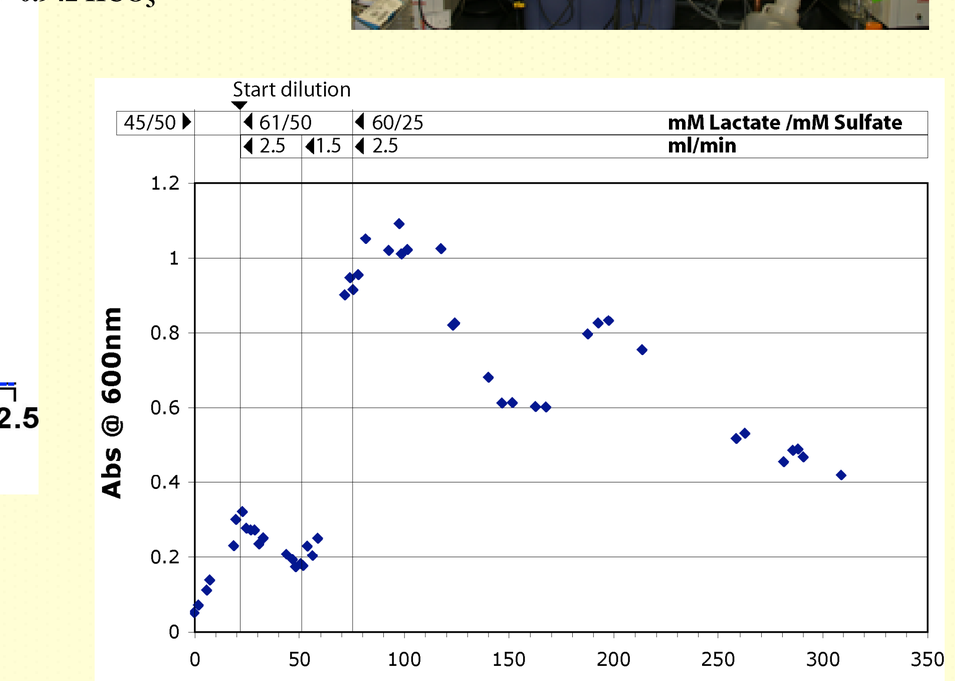


DM indicate co-culture of *Desulfovibrio vulgaris* with *M. maripulidis*
PM indicate co-culture of *Desulfovibrio sp*. PT2 with *M. maripulidis*
Figures C and D depict total amount of µmol of gases in head space of tubes.

The Hydrogen Burst







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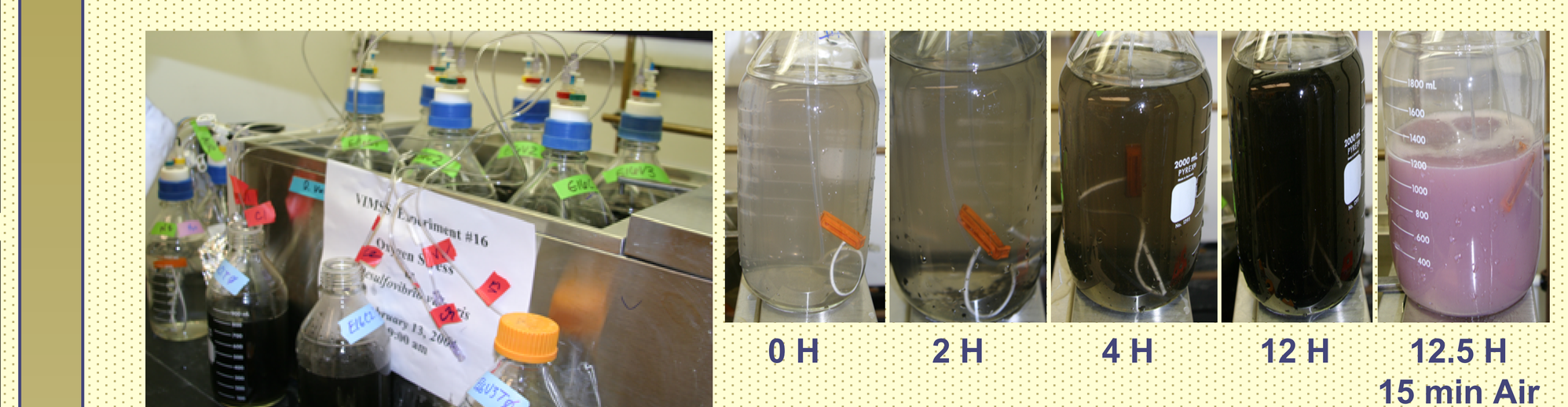
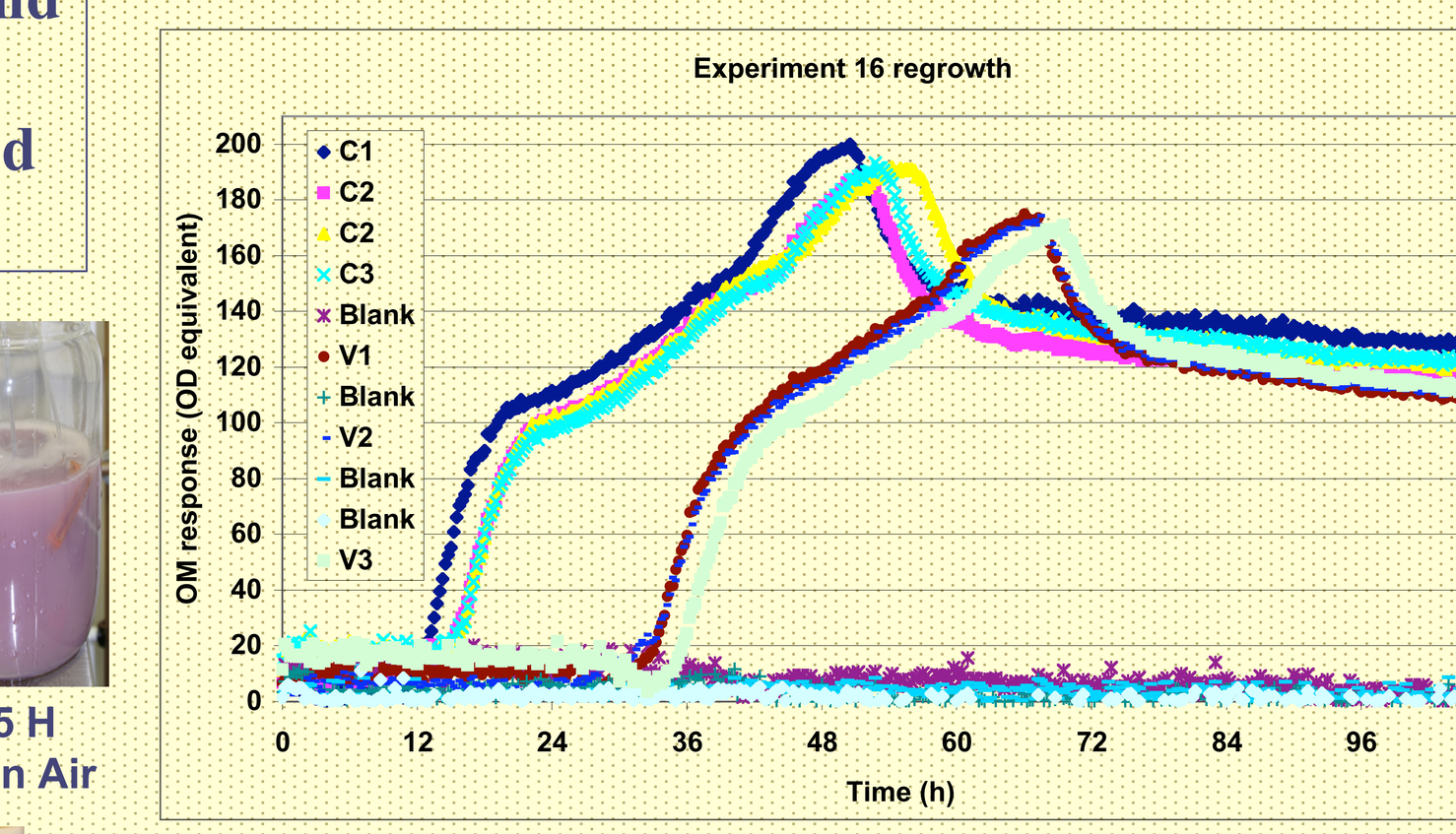
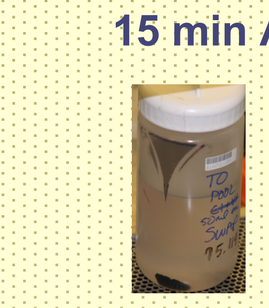
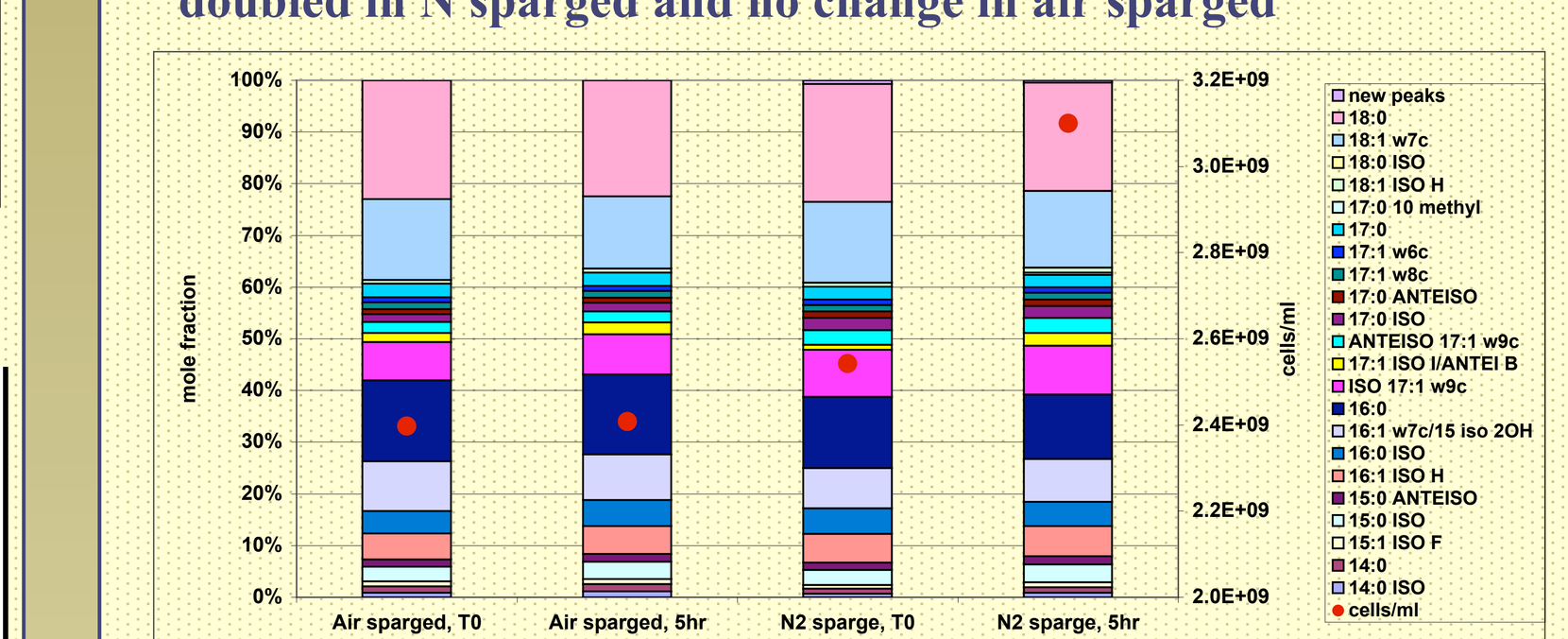
Cells grown batch from log phase starter to mid log phase and then sparged with air and nitrogen for 5 h (one generation time) and harvested at T0 and T5 for 5 VIMSS labs. Aerated cells still recover full growth potential (air has longer lag).

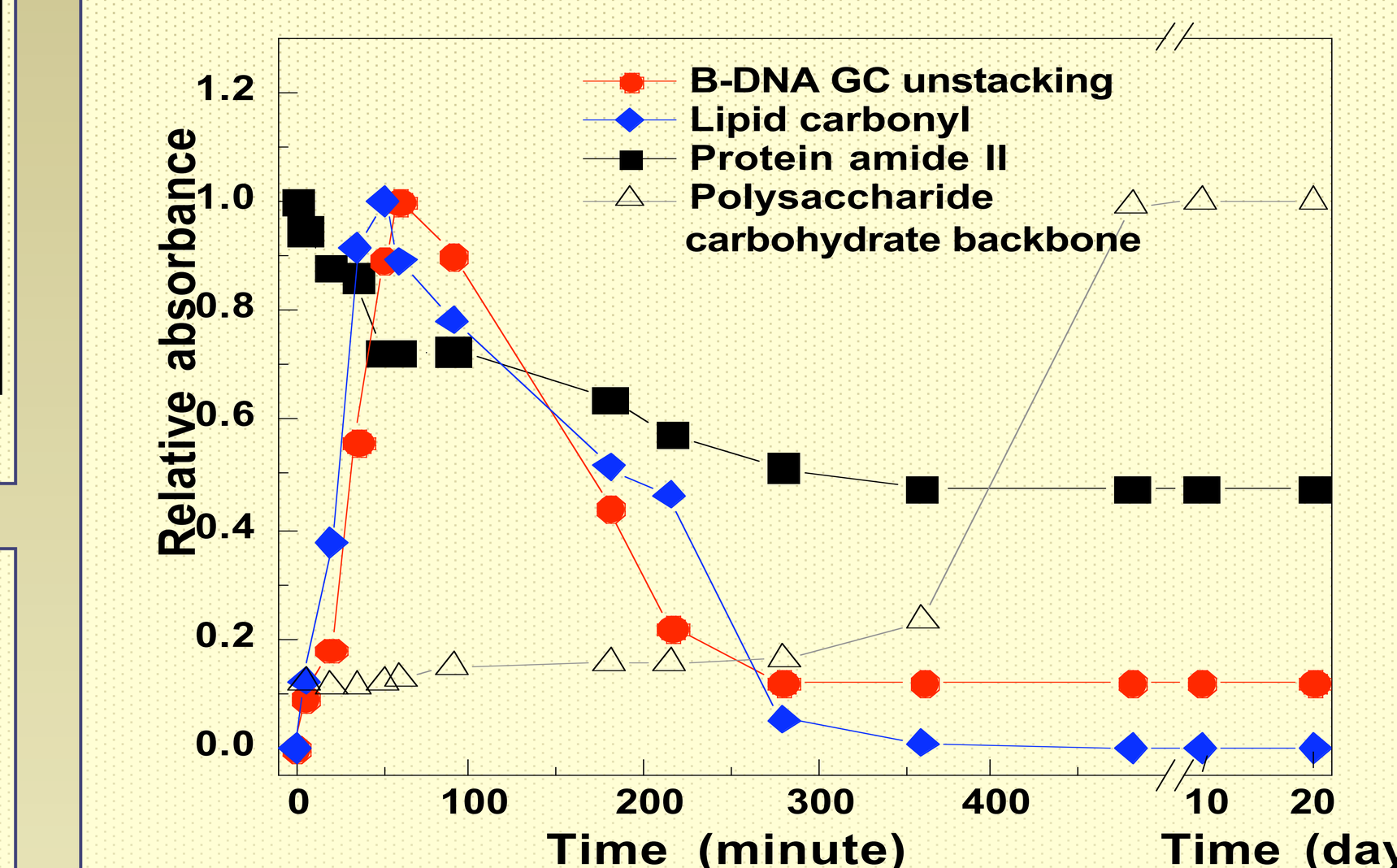




Phospholipid Fatty Acid changes minimal over 5 h, total lipids doubled in N sparged and no change in air sparged

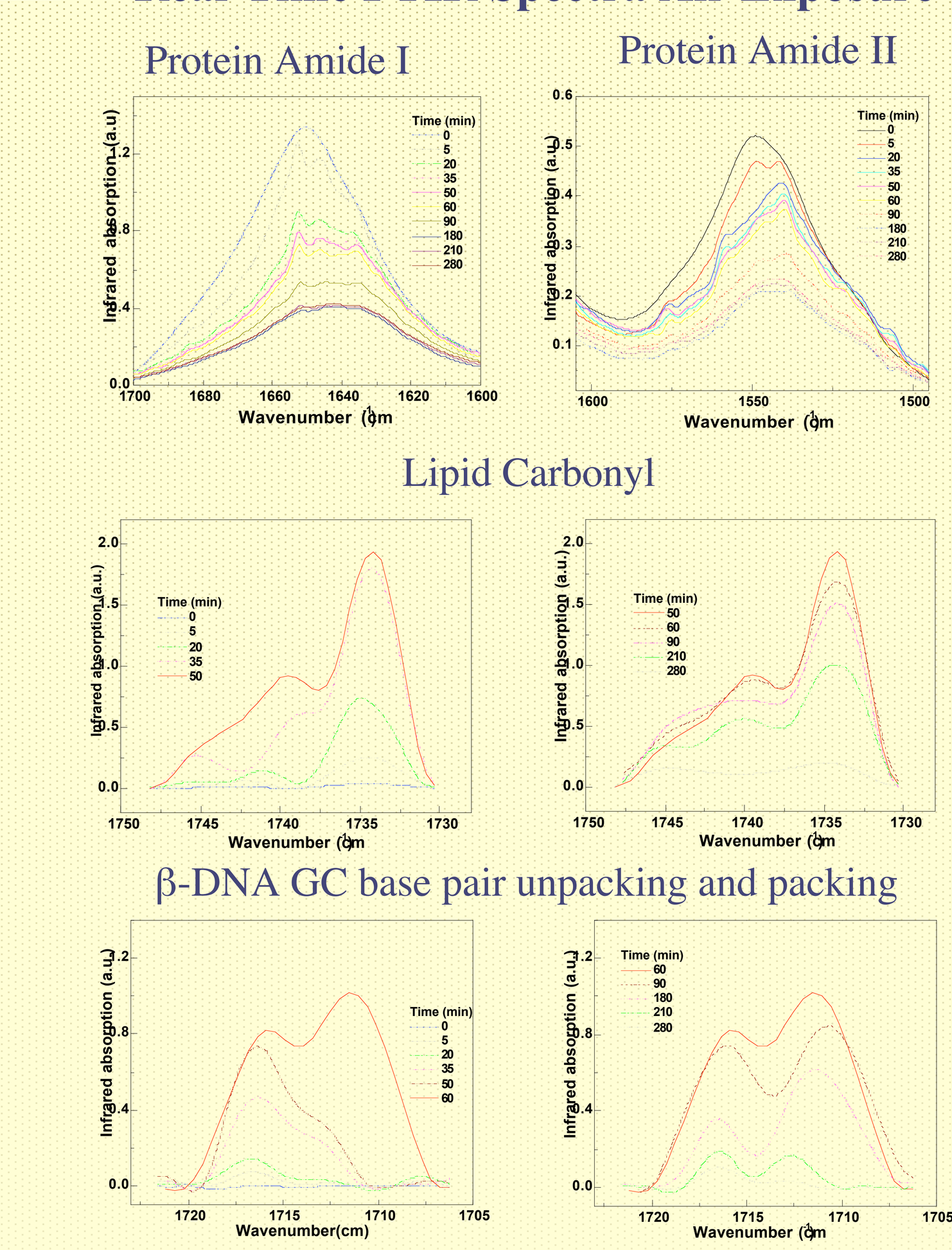


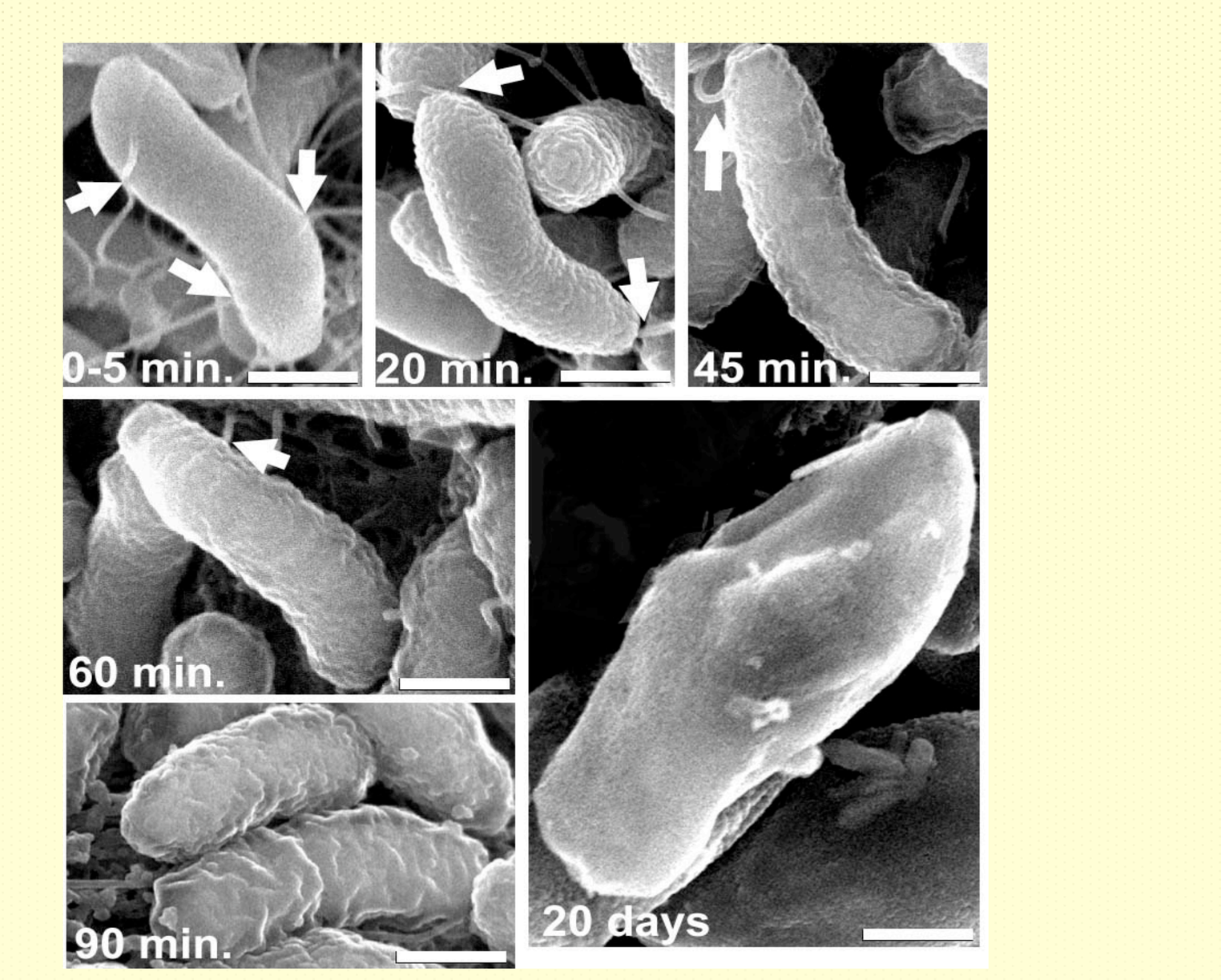


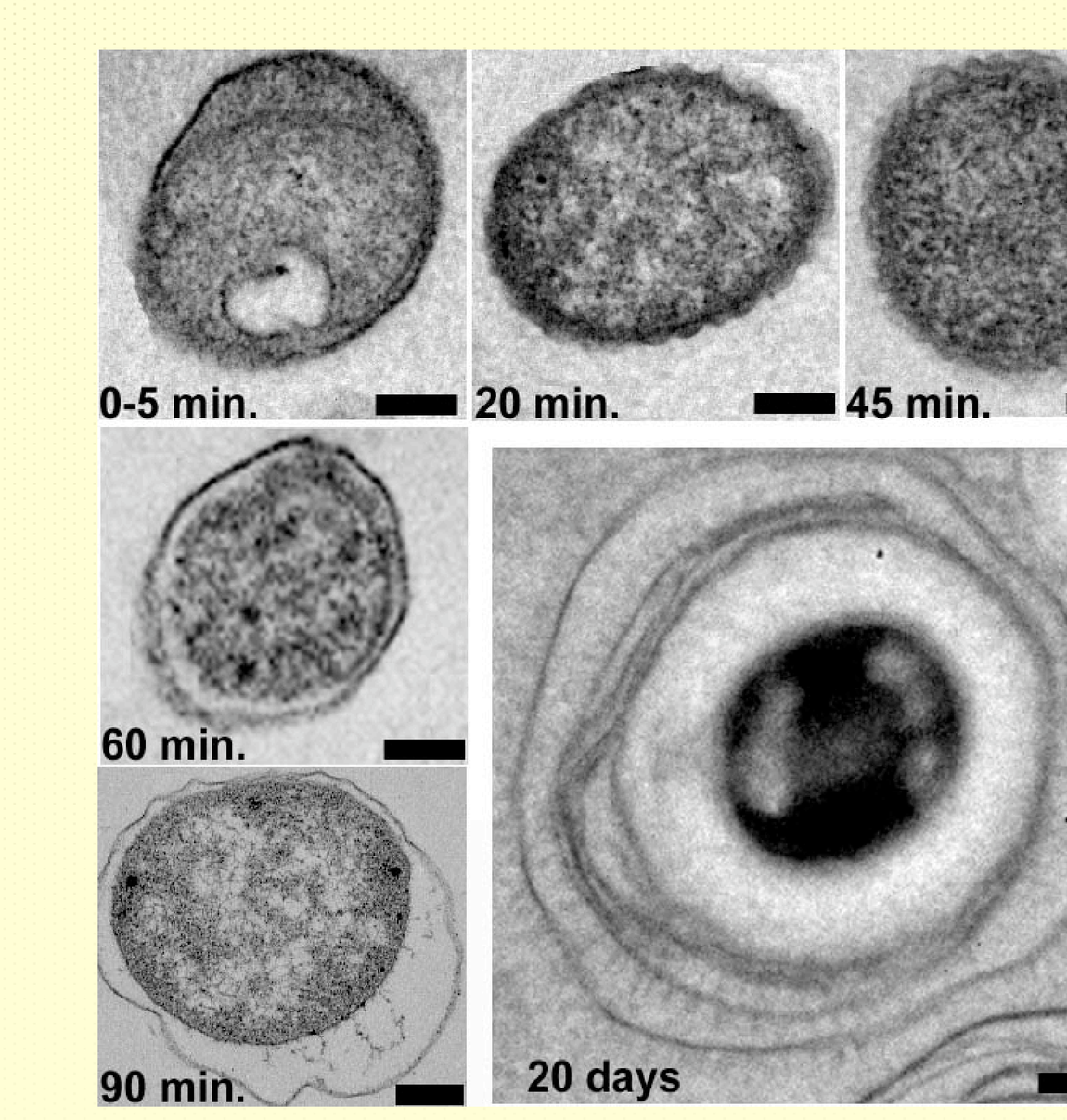


Time course of infrared absorption intensity, indicative of oxidative stress levels in different biologically important molecules in *Desulfovibrio vulgaris* after exposure to atmospheric oxygen.

Real-Time FTIR Spectra Air Exposure







Our working hypothesis is that there are 3 distinct phases that occur in *D. vulgaris* stress response to exposure to Air. The first phase is cell membrane damage and accumulation of protein damage in the cell, formation of protein aggregates in the cytoplasm, and loss of 30-40 % of the proteins. This happens during the first 60 minutes, in the first few minutes they are negatively aerotactic, but by the end of phase 1 of continued air exposure they have lost all of their flagella, even though the motor is still present. During phase 2 *D. vulgaris* begins to secrete an exopolysaccaharide sheath or capsule, and the oxidative stress in the cell begins to level off and this occurs from 60-90 min post exposure. During phase 3 the oxidative stress in terms of protein damage, etc. begins to decrease and the aggregates disappear. This occurs during 90-480 min. After 480 min stress damage is undetectable. During phase 3 the cell wall periplasmic space also thickens. At this point the cells are capable of surviving for many days and are completely recoverable in a matter of hours. This hypothesis and the exact mechanisms are being elucidated by the proteomics, transcriptomics and metabolomics being done by the other VIMSS labs.